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ORIGINAL ARTICLE

Targeted next-generation sequencing detects rare genetic events in pheochromocytoma and paraganglioma

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ABSTRACT

Background Knowing the genetic status of patients affected by paragangliomas and pheochromocytomas (PPGL) is important for the guidance of their management and their relatives. Our objective was to improve the diagnostic performances of PPGL genetic testing by next-generation sequencing (NGS).

Methods We developed a custom multigene panel, which includes 17 PPGL genes and is compatible with both germline and tumour DNA screening. The NGS assay was first validated in a retrospective cohort of 201 frozen tumour DNAs and then applied prospectively to 623 DNAs extracted from leucocytes, frozen or paraffin-embedded PPGL tumours.

Results In the retrospective cohort, the sensitivity of the NGS assay was evaluated at 100% for point and indels mutations and 86% for large rearrangements. The mutation rate was re-evaluated from 65% (132/202) to 78% (156/201) after NGS analysis. In the prospective cohort, NGS detected not only germline and somatic mutations but also co-occurring variants and mosaicism. A mutation was identified in 74% of patients for whom both germline and tumour DNA were available.

Conclusion The analysis of 824 DNAs from patients with PPGL demonstrated that NGS assay significantly improves the performances of PPGL genetic testing compared with conventional methods, increasing the rate of identified mutations and identifying rare genetic mechanisms.

INTRODUCTION

Paragangliomas and pheochromocytomas (PPGL) are rare neuroendocrine tumours that can arise either from the adrenal medulla (pheochromocytoma, PCC) or from extra-adrenal paraganglia (paraganglioma, PGL).¹ Parasympathetic PGLs mainly occur in the head and neck region (HN-PGLs). In contrast, PCCs and sympathetic PGLs arise from the thoracic, abdominal and pelvic areas (TAP-PGLs). PPGLs are considered as the human tumours with the highest frequency of hereditary cases with at least 35% of inherited forms of the disease.² Driver mutations can also be identified at somatic level, and overall, germline or somatic mutations in one of the 18 known genes

involved in PPGL pathogenesis, generally mutually exclusive, are present in about 60% of tumours.³ These PPGL genes include *VHL*, *NF1*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *FH*, *MAX*, *EPAS1*, *HRAS*, *EGLN1*, *ATRX*, *MET*, *MDH2* and *SLC25A11*, demonstrating a high degree of heterogeneity in the genetic determinism of these neoplasms.

Published guidelines on PPGL recommend that genetic testing should be considered in all patients with PPGL.⁴ For years, a sequential strategy prioritising the Sanger genotyping according to clinical feature-driven diagnostic algorithms was generally used. Given the large number of PPGL susceptibility genes and the high mutation rate, the use of next-generation sequencing (NGS) is now indicated as it allows a simultaneous screening of all PPGL genes of interest.² Our objective was to validate a unique custom PPGL gene panel allowing the sequencing by NGS of the main PPGL genes in germline and tumour DNAs, including DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues in order to rapidly identify driver mutations in patients and tumours for guiding follow-up, targeted therapies in case of metastatic PPGL and familial genetic counselling.

METHODS

Patients

The procedures used for PPGL diagnosis were in accordance with both internal and international clinical practice guidelines.^{4,5} Diagnosis was confirmed by histology in every case. All patients provided written informed consent for PPGL genetic testing, collection of samples and subsequent analyses. This work is a part of the COMETE TACTIC study (ClinicalTrials.gov Identifier: NCT02672020).

Validation cohort

The validation cohort is a retrospective cohort comprising 202 previously genotyped DNAs extracted from frozen tumours collected by the French 'Cortico et Médullosurrénale: les Tumeurs Endocrines' (COMETE) network, for which multiomics analysis was already available and published,^{6–11} providing from 190 different and

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consecutive patients. Mutation analysis for *RET*, *VHL*, *SDHB*, *SDHC* and *SDHD* genes was performed by direct sequencing of corresponding germline DNA for each patient. When direct sequencing was negative, *VHL*, *SDHB*, *SDHC* and *SDHD* were also analysed for the presence of large deletions using the Multiplex Ligation-dependent Probe Amplification (MLPA) method (MRC Holland, The Netherlands). Additional Sanger sequencing was partially performed in germline DNAs (*MAX*, *TMEM127*, *FH*, *SDHA* and *SDHAF2* genes) and tumour DNAs (*VHL*, *RET*, *NF1*, *MAX*, *FH*, *SDHx*, *EPAS1* and *HRAS* genes).^{8,9,11} An *SLC25A11* mutation was identified by exome sequencing in one patient whose tumour was included in this cohort.¹² Moreover, the genotyping of hotspot mutations in *FGFR1* exons 12 and 14 was carried out by Sanger sequencing as this gene was not included in the design of the NGS panel. An *FGFR1* hotspot mutation was identified in three tumours (Unpublished data). MAML3-UBTF fusion transcripts were found in four tumours.¹³

Prospective cohort

The prospective cohort was composed by 578 consecutive patients with PPGL diagnosed in 70 different centres to whom PPGL genetic testing has been proposed. Among the 623 analysed DNAs, 578 were germline DNAs extracted from blood sampling and 45 were tumour DNAs (7 extracted from FFPE tissues and 38 extracted from frozen tissues).

Nucleic acid extraction

Germline DNA was extracted from leucocytes using standard protocols by QIAamp DNA Midi kit (Qiagen) or Chemagic DNA Blood kit in combination with a Chemagic 360 equipment (Perkin Elmer). DNA and RNA from frozen tumour tissues were extracted and purified using an Allprep Kit (Qiagen). DNA extraction from FFPE tumour tissue was performed using the Maxwell 16 instrument (Maxwell 16 FFPE Plus LEV DNA purification kit) (Promega).

Custom PPGL gene panel

The targeted gene panel for PPGL 'MASTR Plus SDHv2' was designed using the MASTR Plus technology (Multiplicom, Agilent Technologies) and included *VHL*, *NF1*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *FH*, *MAX*, *EPAS1*, *EGLN1*, *EGLN2*, *MDH2*, *ATRX* and *HRAS* genes. All coding exons were encompassed excepted for *RET* (exons 8 and 10 to 16), *EPAS1* (exons 9 and 12) and *HRAS* (exons 2 to 4). Primers were designed to cover the whole coding sequence and 20bp of intronic flanking regions, allowing for the detection of mutations in splice sites. To face the difficulties to analyse degraded DNA extracted from FFPE samples, the amplicon mean size was designed at 194bp (range 120bp–230bp in length). The 'MASTR Plus SDHv2' panel comprises 451 amplicons designed against the human NCBI GRCh37/hg19 reference genome assembly. DNA libraries were prepared according to the manufacturer's recommendations and 2×150bp paired-end sequenced on MiSeq platform (Illumina, California, USA) using v2 chemistry according to the standard protocol.

The 'MASTR Plus SDHv2' panel and bioinformatics pipeline, described below, were first validated on two DNA samples (NA12878 and NA19240) acquired from Coriell Institute (Coriell Cell Repositories, Camden, New Jersey, USA).

Best coverage was obtained for runs of 24 samples. Nine regions of systematic low coverage were found, likely due to high GC contents or repeated sequences, which may affect primer binding. Low coverage affected 0.4% of targeted regions

(251/61 346pb). Length of uncovered loci is listed in the online supplementary table S1. All the gaps affecting coding sequence or canonical splice sites were sequenced by the Sanger method.

Additional information can be found in the online supplementary methods.

Data analysis

Demultiplexing was performed using MiSeq Reporter (Illumina, California, USA). Alignment and variant calling were performed using SeqNext (JSI Medical Systems) and PolyDiag (Paris Descartes University) software. PolyDiag software used BWA-MEM as a read aligner and Freebayes, Samtools and GATK as variant callers. The 17 genes included in the 'MASTR Plus SDHv2' panel were analysed on tumour DNAs. As, in PPGL, *ATRX* and *HRAS* genes have been reported as somatically mutated only,^{14,15} they were not analysed in germline DNAs of patients. The variant analysis was mainly performed using Alamut Visual 2.7 (Interactive Biosoftware) as an interface. Variant classification into five classes¹⁶ was based on the framework published by the NGSnPPGL group taking into account multiple criteria including the frequency of the variant in the general population and disease databases, its description in the literature, the variant type, the co-segregation with the disease in families if any, the co-occurrence with known pathogenic mutation, the in silico predictions and the results of functional or supplemental studies.²

Additional information can be found in the online supplementary methods.

Sanger sequencing

Regions with <30X depth of coverage were analysed using Sanger sequencing. The presence of all variants of interest (VOI), corresponding to variants of unknown significance (VUS, class 3), likely pathogenic (class 4) or pathogenic (class 5) variants¹⁶ was confirmed using Sanger sequencing except when they were already known for tumours from the retrospective cohort.^{8–11} Variants identified in tumour DNA were systematically searched for in corresponding germline DNA when it was available.

Search for large rearrangements

In the prospective cohort, *VHL*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD* and *MAX* genes were also analysed for the presence of large deletions or duplications using the MLPA method (MRC Holland, The Netherlands).

Immunohistochemistry

To assess the functionality of some VUS, immunostaining was performed when possible. Paraffin blocks were cut and sections (4–6 µm thick) were mounted on Superfrost Plus slides and used for immunohistochemistry as described previously.^{17–19} The antibodies used were anti-SDHB (HPA002868, Sigma-Aldrich, 1/500), anti-SDHA (Abcam, ab14715, 1/1000) and anti-2SC.

Droplet digital PCR

Sample partitioning was performed using the QX200 Droplet Generator (Bio-Rad), PCR amplification using the C1000 Thermal Cycler (Bio-Rad) and droplet reading using the Droplet Reader (QX 200), which provides absolute quantification in digital form. TaqMan probe-based assays (Bio-Rad) were used. Each mutated event was detected with a primer labelled with FAM fluorophore and the corresponding wild-type allele with a primer labelled with HEX fluorophore. Reference of Droplet Digital PCR (ddPCR) Mutation Assay (Bio-Rad) for

the *SDHB* c.557G>A nucleotide variation is the following: dHsaMDS127410807.

RESULTS

Validation of the NGS 'MASTR Plus SDHv2' panel in the retrospective cohort

To assess the diagnostic accuracy of the 'MASTR Plus SDHv2' panel, we first searched for the known mutations previously identified in the validation cohort. Tumour DNA was available for 201 out of the 202 samples of the COMETE collection (online supplementary table S2). Using Sanger sequencing, we had previously identified 119 point or short indel mutations in 116 tumour DNAs (66 mutations at the germline and 53 at the tumour level). All mutations were confirmed by NGS using the 'MASTR Plus SDHv2' panel. A germline *NF1* pathogenic mutation was found in the 10 patients with a clinical diagnosis of neurofibromatosis type 1 (NF1), but who had not benefited from a previous *NF1* molecular testing (online supplementary table S2). Six out of the eight (75%) MLPA-confirmed gross deletions were detected by the NGS assay (two whole *VHL* deletions, one whole *MAX* deletion, one *SDHC* exon 3 deletion, one *SDHB* exon 1 deletion and one exon 3 *VHL* deletion). An *SDHC* exon 2 deletion was not detected due to the absence of coverage of this exon by the NGS panel and a somatic *NF1* whole deletion was missed.

Improvement of mutation detection in the retrospective cohort

Besides the confirmation of the already known mutations, 32 additional VOI were identified using the NGS panel, including unknown VOI in tumours previously considered as 'negative' or additional VOI in mutated-tumours (online supplementary figure S1).

Identification of VOI in 'negative' tumours

A variant of interest was identified in 24 of the 70 tumours previously considered as 'negative' (ie, without mutation identified by former Sanger sequencing). A likely causative mutation was found through the 'MASTR Plus SDHv2' panel in 16 tumours and using the *FGFR1* Sanger sequencing in 2 tumours. Class 3 to 5 variants were identified in *VHL* (n=4), *EPAS1* (n=4), *RET* (n=4), *NF1* (n=3), *FGFR1* (n=2) and *HRAS* (n=1) genes. All but one (*NF1*, p.Leu2650Ser) were present in the tumour DNA only. Moreover, two *ATRX* likely pathogenic variants (c.242+2T>A and c.5114_5122del) were found in two metastatic tumours, while one *ATRX* missense variant c.6869A>G, p.Asn2290Ser was found in a benign tumour at both germline and somatic level. In addition, *UBTF-MAML3* fusions were identified in four tumours.¹³

Identification of additional VOI in mutated tumours

Thirteen additional mutations or VUS were detected using the NGS panel and 1 *FGFR1* mutation using Sanger sequencing. Four somatic mutations (three in *SDHB*-mutated tumours and one in a tumour with biallelic *FH* mutations, all metastatic cases) and one missense VUS (in a benign *VHL*-related case) were identified in the *ATRX* gene. One VUS in *EGLN1* (p.Ser75Phe) was detected as being associated with a somatic *VHL* mutation. One somatic *FGFR1* mutation was found in a PCC of a patient presenting a missense *NF1* germline variant, p.Ala2511Val described as likely benign. Its heterozygosity in the tumour DNA and the occurrence of the somatic *FGFR1* mutation are two additional criteria in favour of the non-pathogenicity of the *NF1* p.Ala2511Val

Figure 1 Distribution of VOI in PPGL susceptibility genes identified by NGS panel in germline DNA of 541 patients. NGS, next-generation sequencing; PPGL, pheochromocytoma and paraganglioma; VOI, variants of interest.

variation. One PCC, carrying a somatic c.25del, p.Val9Trpfs*56 mutation in the *MAX* gene with an allelic frequency of 11%, presented an additional c.182A>G, p.Gln61Arg mutation in *HRAS*, with a higher ratio (35%) revealing a genetic heterogeneity in this tumour. The NGS assay discovered a somatic double hit, corresponding to two different somatic mutations in the same gene, in three tumours (two with *NF1* mutations and one with *VHL* mutations). Finally, three VUS in *SDHA* were found (two in *RET*-mutated tumours and one in an *SDHB*-mutated tumour) with an allelic ratio close to 50%, suggesting that these variants were likely heterozygous. Their association with a driver mutation, their report in the gnomAD database and their non-association with an *SDHA* second hit in tumours are not in favour of their pathogenicity.

Diagnostic performances of the NGS assay in the prospective cohort

Genetic testing of germline DNA

The NGS 'MASTR Plus SDHv2' panel combined with MLPA assays was performed in germline DNA of 541 patients diagnosed with PPGL and identified 120 VOI belonging to class 3 (n=39), class 4 (n=24) and class 5 (n=57) (online supplementary table S3). The most frequent germline mutated genes were *SDHB* (n=30; 5.5% of patients), *SDHA* (n=19; 3.5% of patients), *SDHD* (n=13; 2.4% of patients) and *VHL* (n=11; 2% of patients) (figure 1). The overall germline mutation rate was of 22% (120/541) when considering all the VOI and 15% (81/541) when considering variants from classes 4 and 5 only. Unfortunately, we were not able to retrieve any samples to study the effect of synonymous variants on mRNA splicing.

Interestingly, the NGS assay allowed identifying three patients presented two germline VOI in two different genes. One patient with a PCC diagnosed at 55 years age carried two missense variants, one in *FH* (c.151C>T, p.Arg51Trp) and one in *EPAS1* (c.1635C>G, p.Ile545Met). Moreover, one *SDHB* mutation (c.435del, p.Phe146Serfs*12) associated with an *SDHD* variant of unknown significance (c.158C>T, p.Pro53Leu) was found in a patient with an HN-PGL diagnosed at 26 years age. Finally, two previously described mutations, one in *NF1* (c.2033dup, p.Ile679Aspfs*21) and one in *SDHD* (c.170-1G>T) were identified in a patient who developed both a PCC and a carotid PGL.

Combination of NGS in both germline and tumour DNA

Thirty-seven patients were investigated by NGS assay in both germline (n=37) and tumour (n=45) DNA. VOI were found in 26/37 patients (70%) (online supplementary table S3). Three patients had an *SDHA* germline mutation. One of them, the intronic deletion c.771–21_771–13del, was heterozygous at the germline level (50% of reads) and compatible with a loss of heterozygosity (LOH) in the tumour DNA (85% of reads). The analysis of the corresponding cDNA extracted from leucocytes showed aberrant splicing (exon 7 skipping) demonstrating the pathogenicity of the *SDHA* mutation. In the two other cases, both germline and somatic *SDHA* mutations were identified, leading to a biallelic *SDHA* inactivation in the tumours. The first tumour associated the germline c.1775A>G, p.His592Arg and the somatic c.1753C>T, p.Arg585Trp *SDHA* mutations and the second one, the germline c.16663+3G>C and the somatic c.1765C>T, p.Arg589Trp. All these three tumours exhibited negative SDHA and SDHB immunostaining confirming the deleterious effect of these mutations.²⁰ One patient presented a germline missense VUS in *EGLN2*, c.547G>A, p.Val183Met associated with loss of the wild-type allele (allelic ratio of 48% in the germline and 63% in the tumour DNA). Another patient with a personal and family history of NF1 was found to be a carrier of a germline missense *NF1* mutation c.2903T>G, p.Met968Arg also associated with loss of the normal allele (allelic ratio of 48% in the germline and 81% in the tumour DNA). We also identified a hotspot mutation in *EPAS1* (p.Pro531Arg) in association with an *SDHD* c.158C>T, p.Pro53Leu variant at the germline level. In this tumour, SDHB immunostaining was negative, but there was no evidence for LOH at the *SDHD* locus. Hence, the causative role of each variant in the determinism of this tumour could not be fully determined.

Among tumours with somatic mutations only, one presented biallelic *FH* mutations ([c.364_367del(;);c.1178C>T]). Immunohistochemistry for 2-succinocysteine (2-SC) performed on the tumour tissue showed positive staining which is in favour of

the pathogenicity of both variants. In a patient presenting with metastatic multiple PGLs but no germline mutation, we analysed two different tumours (one PCC and one PGL of Zuckerkandl organ). We identified two different *EPAS1* heterozygous hotspot mutations: one tumour carried the c.1591C>T, p.Pro531Ser and the second one the c.1589C>T, p.Ala530Val mutation. Among other tumours, a single somatic mutation was found in *VHL* (n=7), *HRAS* (n=4) and *EPAS1* (n=3). One heterozygous *SDHB* missense somatic variation c.563T>C, p.Leu188Pro was discovered in a metastatic PCC harbouring a negative SDHB and a positive SDHA immunostaining, which brought evidence for the pathogenicity of the mutation. One *SDHD* frameshift somatic mutation c.284_285insAGGG was detected in a patient with multiple HN-PGLs and lymph node metastases. The allelic ratio of the mutation was 27% in the primary tumour and 35% in the metastasis. Mosaicism was excluded in both cases (allelic ratio for the mutated allele in germline DNA was respectively 0/2205 and 0/1703).

Finally, the NGS 'MASTR Plus SDHv2' panel allowed identifying two mosaic mutations. The *SDHB* c.557G>A, p.Cys186Tyr mutation (allelic ratio=42%) was found in the DNA extracted from a TAP-PGL diagnosed at 24 years age (figure 2A). The search for the mutation in the corresponding germline DNA with the NGS assay revealed the presence of the mutation in 15% of the reads, demonstrating the constitutional mosaicism of the *SDHB* mutation. The presence of the *SDHB* mutation at the mosaic state was confirmed by Sanger sequencing (figure 2B) and droplet digital PCR (figure 2C). Nevertheless, on the electropherogram of Sanger sequencing, the presence of the mutation in the blood DNA would probably have been missed because the peak corresponding to the mutation could be misleadingly considered as an artefact. In a patient who presented a left PCC at 13 years and a right PCC diagnosed 10 years later, a pathogenic *VHL* mutation c.482G>A, p.Arg161Gln was found in the DNA extracted from the left PCC (allelic ratio=24%) and the right PCC (allelic ratio=18%). In the DNA extracted

Figure 2 Identification of the c.557G>A *SDHB* mutation at mosaic state. (A) NGS results (antisense). (B) Electropherogram from Sanger sequencing of *SDHB* exon 6. (C) Quantification of each allele by ddPCR. ddPCR, droplet digital PCR; Mut.Al, mutated allele, n.d, not detected; NGS, next-generation sequencing.

from leucocytes, the mutation was found in 6 out of 1898 reads (0.3%), which was difficult to distinguish from the background noise. Droplet digital PCR experiments revealed 26% of mutated allele in the left PCC, 19% in the right PCC and 0.6% in the blood DNA versus 0.09% in the control DNA (data not shown). These findings are in favour of a low level of germline mosaicism, but the analysis of another tissue (ie, buccal cells) would be helpful to confirm it. The low allelic ratio of mutations in tumour tissues could be explained by the high vascular density of *VHL*-related and *SDHx*-related tumours leading to high level of contamination by non-mutated cells. In this context of allelic fraction <50%, mutations being secondary genetic events rather than primary driver mutations could not be excluded, but their presence in both tumour and germline DNA confirmed the mosaicism.

Altogether, the use of the NGS assay in the prospective cohort allowed finding mutations at germline, somatic and mosaic levels (online supplementary table S3). All variants reported by NGS were validated by Sanger sequencing, revealing no false positive. The NGS panel also accurately detected 10 large deletions, which were all confirmed by MLPA.

DISCUSSION

Our results demonstrate that the NGS assay with the custom-designed targeted 'MASTR Plus SDHv2' panel significantly improves the diagnostic accuracy for mutation detection in PPGL genes compared with former technical approaches. Several studies previously reported the use of targeted NGS assays for PPGL genetic testing, including various genes.^{21–24} Recently, the NGSnPPGL study group adopted a modified version of ClinVar's 'gold star' scale to assign an evidence level to each gene reported as implicated in the genetics of PPGL.² Level 3 and 4 genes comprised those with established evidence or functional validation of involvement in PPGL tumorigenesis at the germline or somatic level. The advantage of our assay, compared with others previously published, is to combine a comprehensive gene panel that includes all the level 3 and 4 genes defined in the consensus statement on PPGL² to an optimised design allowing analysing germline, frozen tumour DNA and DNA from FFPE tumour tissues in a single assay. The sensitivity of the panel was assessed on 119 point mutations or short indels previously found by Sanger sequencing in the COMETE cohort and reached 100% (online supplementary table S2). Our pipeline was able to easily detect indels, including a 37bp deletion in the *NF1* gene. Considering large rearrangements affecting exons with good coverage, the sensitivity was 86% (6/7). The only gross deletion missed involved the whole *NF1* gene and was the unique somatic large deletion found in the cohort. This lack of detection was likely due to important contamination of the analysed tumour tissue by normal cells.

Altogether, the mutation rate in the validation cohort, previously assessed to 65% (132/202) by Sanger sequencing, was re-evaluated at 78% (156/201) after NGS assay analysis, *FGFR1* Sanger sequencing and MAML3-UBTF fusion transcript search for (online supplementary figure S1). Among variants identified by NGS, seven had an allelic ratio <20% explaining why Sanger sequencing could miss them. The other ones were found either in genes not previously genotyped by Sanger (ie, *ATRX*) or because previous genotyping was done partially only (ie, genotyping limited to tumours presenting a specific transcriptomic or LOH profile). It is noteworthy that some somatic events with low allelic fraction may not be pathogenically relevant especially when they do not affect clearly established PPGL susceptibility

genes. Interestingly, the new genetic data generated by NGS clarify the classification of the PPGL COMETE collection after transcriptomic analysis.^{8,9} All tumours classified into the cluster C1B, which was defined as the *VHL*-related cluster, were explained by either a *VHL* (26 germlines and 15 somatic) or an *EPAS1* mutation (1 case). In the tumours classified in the cluster C2A (defined as the *RET/NF1* cluster), mutations were identified in 64/78 (82%), including 36 in *NF1*, 16 in *RET*, 7 in *HRAS*, 3 in *FGFR1* and 1 in *TMEM127* and *MAX*. In the cluster 2B-related tumours, four somatic *EPAS1* mutations and four MAML3-UBTF fusion transcripts were detected.

Considering the prospective cohort, VOI have been found in all except one (*SDHAF2*) gene belonging to the 'MASTR Plus SDHv2' panel validating the accuracy of the selected gene list (figure 1). Given that the mutation rate is of 15% (class 4 and 5 variants) in patients with an apparently sporadic presentation of the disease (ie, a single benign tumour without any history of the disease or syndromic presentation, 406/541) in our prospective cohort, our data are in accordance with previous publications reporting a mutation rate of 11%–13% in patients with sporadic PPGL.^{24,25} In patients with a family history of PPGL (n=9), a mutation was found in 100% of cases (four in *VHL*, two in *SDHD*, one in *SDHB*, one in *SDHC* and one in *MAX* gene). In all six patients with personal or familial clinical presentation evocating NF1, the causative *NF1* mutation was identified. In patients with multiple or recurrent PGLs (n=21), germline mutations were found in 12 patients (57%), all in *SDHx* genes and interestingly, affected each *SDHx* gene (*SDHA*, *SDHB*, *SDHC* and *SDHD*). In patients with a single HN-PGL, mutations were mainly found in *SDHx* genes (10 in *SDHA*, 10 in *SDHB*, 6 in *SDHC* and 8 in *SDHD*) but class 3 variants were also found in *EGLN2*, *FH* and *TMEM127*, suggesting their potential role in the development of parasympathetic PGLs and justifying their genotyping in patients with HN-PGL only.

A remarkable finding was the identification of two germline pathogenic mutations, affecting *SDHD* and *NF1*, in a patient who developed multiple tumours (PCC and carotid PGL). The *SDHD* (c.170–1G>T) mutation was previously reported as leading to exon 3 skipping²⁶ and responsible for familial PPGL.^{26–29} The *NF1* (p.Ile679Aspfs*21) mutation was also reported several times in patients with neurofibromatosis type 1.³⁰ The retrospective clinical examination of the patient did not reveal any clinical signs of NF1, which is in accordance with the recent description of pathogenic *NF1* mutations in patients with PPGL unsuspecting for NF1.³¹ For this patient, tumour tissue analyses showed a loss of *SDHB* immunostaining, a LOH at the *SDHD* locus and, surprisingly, the loss of the *NF1* allele carrying the *NF1* mutation. All these data support the role of the *SDHD* mutation in the development of patient's tumours and lead to consider the *NF1* mutation as an incidental finding, without direct consequence in the current patient's disease but suggesting that the patient should be screened for NF1 disease. Consequently, presymptomatic testing should be offered to first-degree relatives for both mutations.

Regarding the patient carrying two missense germline variants, one in *FH* (c.151C>T, p.Arg51Trp) and one in *EPAS1* (c.1635C>G, p.Ile545Met), the PCC tissue analysis revealed a LOH at the *FH* locus and positive 2-SC immunostaining, which are both in favour of the *FH*, p.Arg51Trp pathogenicity that can be, then, classified as pathogenic. No additional support was provided regarding the *EPAS1*, p.Ile545Met which is classified as VUS.

No surgery was performed for the patient associating an *SDHB* mutation (c.435del, p.Phe146Serfs*12) and an *SDHD*

Figure 3 Distribution of VOI in PPGL susceptibility genes identified by NGS panel in 227 patients for whom both germline and tumour DNAs were analysed. NGS, next-generation sequencing; PPGL, pheochromocytoma and paraganglioma; VOI, variants of interest.

variant of unknown significance (c.158C>T, p.Pro53Leu) preventing tumour analysis. However, the association of *SDHD* p.Pro53Leu variant with another pathogenic *SDHx* mutation was already described,³² and experimental studies in yeast have shown that this variant does not alter protein function³³ suggesting that *SDHD* p.Pro53Leu could be a rare non-functional polymorphism.

Combining the prospective and the retrospective cohorts, we analysed both germline and tumour DNAs in 227 cases. Among them, 168 (74%) carried a driver mutation: 80 germline (36%) and 86 somatic (37%). The most frequent germline mutated genes were *VHL* (n=25), *SDHB* (n=16), *NF1* (n=15) and *RET* (n=9), whereas somatic mutations were mainly found in *NF1* (n=25), *VHL* (n=20), *HRAS* (n=13), *RET* (n=9) and *EPAS1* (n=9) (figure 3).

Somatic *VHL* mutations were identified in 13/190 (6.8%) cases of the retrospective cohort and in 7/37 (19%) cases of the prospective cohort. This ratio of somatic mutations is higher than in the TCGA cohort (1.7%)³⁴ but comparable to the one reported by Curras-Freixes *et al* (7%).²⁴ Nevertheless, it is worth noting that this proportion is probably slightly overestimated as the prospective cohort analysed at the somatic level was enriched in suspicious cases (young age of onset, multiple locations...).

ATRX variants were found in 3.5% of cases (8/227). Among them, seven were identified at somatic level including one in benign *VHL*-related tumour and six in metastatic cases (three *SDHB*-mutated, one *FH*-mutated and two non-mutated tumours¹³), which is in accordance with the described role of *ATRX* in the development of aggressive PPGL.^{13 15} Interestingly, *ATRX* somatic variants were already reported in benign *VHL* tumour.^{15 35} Among the malignant tumours analysed at both germline and somatic level, 18% (6/33) carried an *ATRX* variant which is slightly lower than the previously reported prevalence (3/12, 25%).³⁴

Interestingly, the patient presenting two different somatic *EPAS1* mutations in two different tumours was diagnosed with a heterozygous haemoglobin C disease (haemoglobin C=32%, haemoglobin A2=2.9%). Although this trait has no clinical or biological expression, it could lead to a chronic hypoxic status driving chromaffin cells more sensitive to *EPAS1* activating

mutations and promoting tumorigenesis, as previously described in patients with cyanotic congenital heart disease.³⁶

Remarkably, this approach identified two *SDHx* somatic mutations (one in *SDHB* and one in *SDHD*), which were rarely reported.^{37 38} Of note, these *SDHx* somatic mutations were both present in patients with metastatic PPGL.

This NGS assay was able to detect two mosaic mutations, one in *SDHB* and one in the *VHL* gene, in patients with young age of onset. To our knowledge, this is the first report of an *SDHB* mosaic mutation. *VHL* mosaicism is better described but usually found in patients with typical *VHL* disease-associated syndromic lesions.^{39 40} In the present case, the patient was diagnosed with bilateral PCC only. The high suspicion for germline mosaicism justifies a complete clinical screening for *VHL* disease. Our results suggest that mosaicism can occur in patients with PPGL (1% of cases in this cohort), and not only in *VHL* or *EPAS1* genes. It was probably underestimated until now due to the low sensitivity of classical sequencing techniques. The current spread of NGS technology use should allow evaluating its prevalence in patients with PPGL. This study demonstrates that deep coverage NGS, combined with ddPCR, allows to discriminate mosaic mutations in patients with PPGL, which is of major importance for family counseling and risk assessment for disease recurrence.

In total, the NGS 'MASTR Plus SDHv2' panel assessed in both retrospective and prospective cohorts, encompassing 768 patients' germline and/or tumour DNA, clearly showed higher accuracy for PPGL genetic testing than conventional methods with a significant increase of the mutation rate. Furthermore, the NGS panel brought an additional value in diagnosing co-occurring variants and mosaicism, both genetic mechanisms not easily identified so far.

The mutational data are essential for guiding genetic counseling, which indicates or exempts (in the presence or the absence of a germline mutation) specific management and follow-up as well as predictive genetic testing in the relatives or to reassure (in front of truly somatic mutations) patients about the risk of relapse.

Finally, the recent knowledge delivered by -omics studies in PPGL molecular pathogenesis gave evidence that patients with metastatic PPGL would benefit from personalised medicine,³ but a precise molecular classification of tumours will be mandatory for guiding targeted therapies. For this purpose, the custom-designed 'MASTR Plus SDHv2' targeted panel would be a relevant molecular tool to quickly assess the molecular portrait of tumours by NGS, even when FFPE-DNA is only available.

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REFERENCES

- Lam AK. Update on Adrenal Tumours in 2017 World Health Organization (WHO) of Endocrine Tumours. *Endocr Pathol* 2017;28:213–27.
- Toledo RA, Burnichon N, Cascon A, Benn DE, Bayley JP, Welander J, Tops CM, Firth H, Dwight T, Ercolino T, Mannelli M, Opocher G, Clifton-Bligh R, Gimm O, Maher ER, Robledo M, Gimenez-Roqueplo AP, Dahia PLM. Consensus Statement on next-generation-sequencing-based diagnostic testing of hereditary pheochromocytomas and paragangliomas. *Nat Rev Endocrinol* 2017;13:233–47.
- Favier J, Amar L, Gimenez-Roqueplo AP. Paraganglioma and pheochromocytoma: from genetics to personalized medicine. *Nat Rev Endocrinol* 2015;11:101–11.
- Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, Naruse M, Pacak K, Young WF. Endocrine Society. Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 2014;99:1915–42.
- Plouin PF, Amar L, Dekkers OM, Fassnacht M, Gimenez-Roqueplo AP, Lenders JW, Lussay-Lepoutre C, Steichen O. Guideline Working Group. European Society of Endocrinology Clinical Practice Guideline for long-term follow-up of patients operated on for a pheochromocytoma or a paraganglioma. *Eur J Endocrinol* 2016;174:G1–G10.
- Favier J, Brière JJ, Burnichon N, Rivière J, Vescovo L, Benit P, Giscos-Douriez I, De Reyniès A, Bertherat J, Badoual C, Tissier F, Amar L, Libé R, Plouin PF, Jeunemaitre X, Rustin P, Gimenez-Roqueplo AP. The Warburg effect is genetically determined in inherited pheochromocytomas. *PLoS One* 2009;4:e7094.
- Burnichon N, Brière JJ, Libé R, Vescovo L, Rivière J, Tissier F, Jouanno E, Jeunemaitre X, Béné P, Tzagoloff A, Rustin P, Bertherat J, Favier J, Gimenez-Roqueplo AP. SDHA is a tumor suppressor gene causing paraganglioma. *Hum Mol Genet* 2010;19:3011–20.
- Burnichon N, Vescovo L, Amar L, Libé R, de Reyniès A, Venisse A, Jouanno E, Laurendeau I, Parfait B, Bertherat J, Plouin PF, Jeunemaitre X, Favier J, Gimenez-Roqueplo AP. Integrative genomic analysis reveals somatic mutations in pheochromocytoma and paraganglioma. *Hum Mol Genet* 2011;20:3974–85.
- Burnichon N, Buffet A, Parfait B, Letouze E, Laurendeau I, Lorient C, Pasmant E, Abernill N, Valeyrie-Allanore L, Bertherat J, Amar L, Vidaud D, Favier J, Gimenez-Roqueplo AP. Somatic NF1 inactivation is a frequent event in sporadic pheochromocytoma. *Hum Mol Genet* 2012;21:5397–405.
- Letouze E, Martinelli C, Lorient C, Burnichon N, Abernill N, Ottolenghi C, Janin M, Menara M, Nguyen AT, Benit P, Buffet A, Marcaillou C, Bertherat J, Amar L, Rustin P, De Reyniès A, Gimenez-Roqueplo AP. Multi-omics analysis defines core genomic alterations in paraganglioma. *Cancer Cell* 2013;23:739–52.
- Castro-Vega LJ, Letouze E, Burnichon N, Buffet A, Disderot PH, Khalifa E, Lorient C, Elaroui N, Morin A, Menara M, Lepoutre-Lussey C, Badoual C, Sibony M, Dousset B, Libé R, Zinzindohoue F, Plouin PF, Bertherat J, Amar L, de Reyniès A, Favier J, Gimenez-Roqueplo AP. Multi-omics analysis defines core genomic alterations in pheochromocytomas and paragangliomas. *Nat Commun* 2015;6:6044.
- Buffet A, Morin A, Castro-Vega LJ, Habarou F, Lussey-Lepoutre C, Letouze E, Lefebvre H, Guilhem I, Haissaguerre M, Raingeard I, Padilla-Girola M, Tran T, Tcharr L, Bertherat J, Amar L, Ottolenghi C, Burnichon N, Gimenez-Roqueplo AP, Favier J. Germline Mutations in the Mitochondrial 2-Oxoglutarate/Malate Carrier *SLC25A11* Gene Confer a Predisposition to Metastatic Paragangliomas. *Cancer Res* 2018;78:1914–22.
- Job S, Draskovic I, Burnichon N, Buffet A, Cros J, Lépine C, Venisse A, Robidel E, Verkarre V, Meatchi T, Sibony M, Amar L, Bertherat J, de Reyniès A, Londoño-Vallejo A, Favier J, Castro-Vega LJ, Gimenez-Roqueplo AP. Telomerase Activation and ATRX Mutations Are Independent Risk Factors for Metastatic Pheochromocytoma and Paraganglioma. *Clin Cancer Res* 2019;25:760–70.
- Crona J, Delgado Verdugo A, Maharjan R, Ståhlberg P, Granberg D, Hellman P, Björklund P. Somatic mutations in H-RAS in sporadic pheochromocytoma and paraganglioma identified by exome sequencing. *J Clin Endocrinol Metab* 2013;98:E1266–71.
- Fishbein L, Khare S, Wubbenhorst B, DeSloover D, D'Andrea K, Merrill S, Cho NW, Greenberg RA, Else T, Montone K, LiVolsi V, Fraker D, Daber R, Cohen DL, Nathanson KL. Whole-exome sequencing identifies somatic ATRX mutations in pheochromocytomas and paragangliomas. *Nat Commun* 2015;6:6140.

- 16 Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV. IARC Unclassified Genetic Variants Working Group. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* 2008;29:1282–91.
- 17 van Nederveen FH, Gaal J, Favier J, Korpershoek E, Oldenburg RA, de Bruyn EM, Sleddens HF, Derckx P, Riviere J, Dannenberg H, Petri BJ, Komminoth P, Pacak K, Hop WC, Pollard PJ, Mannelli M, Bayley JP, Perren A, Niemann S, Verhofstad AA, de Bruine AP, Maher ER, Tissier F, Méatchi T, Badoual C, Bertherat J, Amar L, Alataki D, Van Marck E, Ferrau F, François J, de Herder WW, Peeters MP, van Linge A, Lenders JW, Gimenez-Roqueplo AP, de Krijger RR, Dinjens WN. An immunohistochemical procedure to detect patients with paraganglioma and pheochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncol* 2009;10:764–71.
- 18 Korpershoek E, Favier J, Gaal J, Burnichon N, van Gessel B, Oudijk L, Badoual C, Gadessaud N, Venisse A, Bayley JP, van Dooren MF, de Herder WW, Tissier F, Plouin PF, van Nederveen FH, Dinjens WN, Gimenez-Roqueplo AP, de Krijger RR. SDHA immunohistochemistry detects germline SDHA gene mutations in apparently sporadic paragangliomas and pheochromocytomas. *J Clin Endocrinol Metab* 2011;96:E1472–6.
- 19 Castro-Vega LJ, Buffet A, De Cubas AA, Cascón A, Menara M, Khalifa E, Amar L, Azriel S, Bourdeau I, Chabre O, Currás-Freixes M, Franco-Vidal V, Guillaud-Bataille M, Simian C, Morin A, Letón R, Gómez-Graña A, Pollard PJ, Rustin P, Robledo M, Favier J, Gimenez-Roqueplo AP. Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum Mol Genet* 2014;23:2440–6.
- 20 Papatomas TG, Oudijk L, Persu A, Gill AJ, van Nederveen F, Tischler AS, Tissier F, Volante M, Matias-Guiu X, Smid M, Favier J, Rapizzi E, Libe R, Currás-Freixes M, Aydin S, Huynh T, Lichtenauer U, van Berkel A, Canu L, Domingues R, Clifton-Bligh RJ, Bialas M, Vikkula M, Baretton G, Papotti M, Nesli G, Badoual C, Pacak K, Eisenhofer G, Timmers HJ, Beuschlein F, Bertherat J, Mannelli M, Robledo M, Gimenez-Roqueplo AP, Dinjens WN, Korpershoek E, de Krijger RR. SDHB/SDHA immunohistochemistry in pheochromocytomas and paragangliomas: a multicenter interobserver variation analysis using virtual microscopy: a Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T). *Mod Pathol* 2015;28:807–21.
- 21 Rattenberry E, Vialard L, Yeung A, Bair H, McKay K, Jafri M, Canham N, Cole TR, Denes J, Hodgson SV, Irving R, Izatt L, Korbonits M, Kumar AV, Lalloo F, Morrison PJ, Woodward ER, Macdonald F, Wallis Y, Maher ER. A comprehensive next generation sequencing-based genetic testing strategy to improve diagnosis of inherited pheochromocytoma and paraganglioma. *J Clin Endocrinol Metab* 2013;98:E1248–56.
- 22 Welander J, Andreasson A, Juhlin CC, Wiseman RW, Bäckdahl M, Höög A, Larsson C, Gimm O, Söderkvist P. Rare germline mutations identified by targeted next-generation sequencing of susceptibility genes in pheochromocytoma and paraganglioma. *J Clin Endocrinol Metab* 2014;99:E1352–60.
- 23 Pillai S, Gopalan V, Lo CY, Liew V, Smith RA, Lam AK. Silent genetic alterations identified by targeted next-generation sequencing in pheochromocytoma/paraganglioma: A clinicopathological correlations. *Exp Mol Pathol* 2017;102:41–6.
- 24 Currás-Freixes M, Piñeiro-Yañez E, Montero-Conde C, Apellániz-Ruiz M, Calsina B, Mancikova V, Remacha L, Richter S, Ercolino T, Rogowski-Lehmann N, Deutschbein T, Calatayud M, Guadalix S, Álvarez-Escolá C, Lamas C, Aller J, Sastre-Marcos J, Lázaro C, Galofré JC, Patiño-García A, Meoro-Avilés A, Balmaña-Gelpi J, De Miguel-Novoa P, Balbín M, Matias-Guiu X, Letón R, Inglada-Pérez L, Torres-Pérez R, Roldán-Romero JM, Rodríguez-Antona C, Flíedner SMJ, Opocher G, Pacak K, Korpershoek E, de Krijger RR, Vroonen L, Mannelli M, Fassnacht M, Beuschlein F, Eisenhofer G, Cascón A, Al-Shahrour F, Robledo M. PheoSeq: A Targeted Next-Generation Sequencing Assay for Pheochromocytoma and Paraganglioma Diagnostics. *J Mol Diagn* 2017;19:575–88.
- 25 Brito JP, Asi N, Bancos I, Gionfriddo MR, Zeballos-Palacios CL, Leppin AL, Undavalli C, Wang Z, Domecq JP, Prustsky G, Elraiyah TA, Prokop LJ, Montori VM, Murad MH. Testing for germline mutations in sporadic pheochromocytoma/paraganglioma: a systematic review. *Clin Endocrinol* 2015;82:338–45.
- 26 Renard L, Godfraind C, Boon LM, Vikkula M. A novel mutation in the SDHD gene in a family with inherited paragangliomas—implications of genetic diagnosis for follow up and treatment. *Head Neck* 2003;25:146–51.
- 27 Benn DE, Gimenez-Roqueplo AP, Reilly JR, Bertherat J, Burgess J, Byth K, Croxson M, Dahia PL, Elston M, Gimm O, Henley D, Herman P, Murday V, Niccoli-Sire P, Pasieka JL, Rohrer V, Tucker K, Jeunemaitre X, Marsh DJ, Plouin PF, Robinson BG. Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. *J Clin Endocrinol Metab* 2006;91:827–36.
- 28 Mannelli M, Simi L, Ercolino T, Gaglianò MS, Becherini L, Vinci S, Sestini R, Gensini F, Pinzani P, Mascali M, Guerrini L, Pratesi C, Nesi G, Torti F, Cipollini F, Bernini GP, Genuardi M. SDH mutations in patients affected by paraganglioma syndromes: a personal experience. *Ann NY Acad Sci* 2006;1073:183–9.
- 29 Persu A, Hamoir M, Grégoire V, Garin P, Duvivier E, Reyckel H, Chantrain G, Mortier G, Mourad M, Maiter D, Vikkula M. High prevalence of SDHB mutations in head and neck paraganglioma in Belgium. *J Hypertens* 2008;26:1395–401.
- 30 Heim RA, Kam-Morgan LN, Binnie CG, Corns DD, Cayouette MC, Farber RA, Aylsworth AS, Silverman LM, Luce MC. Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. *Hum Mol Genet* 1995;4:975–81.
- 31 Gieldon L, Masjkur JR, Richter S, Därr R, Lahera M, Aust D, Zeugner S, Rump A, Hackmann K, Tzschach A, Januszewicz A, Prejbisz A, Eisenhofer G, Schrock E, Robledo M, Klink B. Next-generation panel sequencing identifies *NF1* germline mutations in three patients with pheochromocytoma but no clinical diagnosis of neurofibromatosis type 1. *Eur J Endocrinol* 2018;178:K1–9.
- 32 Leidenz FB, Bastos-Rodrigues L, Oliveira M, Mamede M, Sarquis M, Friedman E, de Marco L. Malignant phenotype and two SDHD mutations in a family with paraganglioma syndrome type 1. *Genet Res* 2015;97:e3.
- 33 Panizza E, Ercolino T, Mori L, Rapizzi E, Castellano M, Opocher G, Ferrero I, Neumann HP, Mannelli M, Goffrini P. Yeast model for evaluating the pathogenic significance of SDHB, SDHC and SDHD mutations in PHEO-PGL syndrome. *Hum Mol Genet* 2013;22:804–15.
- 34 Fishbein L, Leshchiner I, Walter V, Danilova L, Robertson AG, Johnson AR, Lichtenberg TM, Murray BA, Ghayee HK, Else T, Ling S, Jefferys SR, de Cubas AA, Wenz B, Korpershoek E, Amelio AL, Makowski L, Rathmell WK, Gimenez-Roqueplo AP, Giordano TJ, Asa SL, Tischler AS, Pacak K, Nathanson KL, Wilkerson MD. Cancer Genome Atlas Research Network. Comprehensive Molecular Characterization of Pheochromocytoma and Paraganglioma. *Cancer Cell* 2017;31:181–93.
- 35 Toledo RA, Qin Y, Cheng ZM, Gao Q, Iwata S, Silva GM, Prasad ML, Ocal IT, Rao S, Aronin N, Barontini M, Bruder J, Reddick RL, Chen Y, Aguiar RC, Dahia PL. Recurrent Mutations of Chromatin-Remodeling Genes and Kinase Receptors in Pheochromocytomas and Paragangliomas. *Clin Cancer Res* 2016;22:2301–10.
- 36 Vaidya A, Flores SK, Cheng ZM, Nicolas M, Deng Y, Opatowsky AR, Lourenço DM, Barletta JA, Rana HQ, Pereira MA, Toledo RA, Dahia PLM. EPAS1 Mutations and Paragangliomas in Cyanotic Congenital Heart Disease. *N Engl J Med* 2018;378:1259–61.
- 37 van Nederveen FH, Korpershoek E, Lenders JW, de Krijger RR, Dinjens WN. Somatic SDHB mutation in an extraadrenal pheochromocytoma. *N Engl J Med* 2007;357:306–8.
- 38 Currás-Freixes M, Inglada-Pérez L, Mancikova V, Montero-Conde C, Letón R, Comino-Méndez I, Apellániz-Ruiz M, Sánchez-Barroso L, Aguirre Sánchez-Covisa M, Alcázar V, Aller J, Álvarez-Escolá C, Andia-Melero VM, Azriel-Mira S, Calatayud-Gutiérrez M, Díaz JA, Díez-Hernández A, Lamas-Oliveira C, Marazuela M, Matias-Guiu X, Meoro-Avilés A, Patiño-García A, Pedrinaci S, Riesco-Eizaguirre G, Sábado-Álvarez C, Sáez-Villaverde R, Sainz de Los Terreros A, Sanz Guadarrama Ó, Sastre-Marcos J, Scolá-Yurrita B, Segura-Huerta Á, Serrano-Corredor ML, Villar-Vicente MR, Rodríguez-Antona C, Korpershoek E, Cascón A, Robledo M. Recommendations for somatic and germline genetic testing of single pheochromocytoma and paraganglioma based on findings from a series of 329 patients. *J Med Genet* 2015;52:647–56.
- 39 Sgambati MT, Stolle C, Choyke PL, Walther MM, Zbar B, Linehan WM, Glenn GM. Mosaicism in von Hippel-Lindau disease: lessons from kindreds with germline mutations identified in offspring with mosaic parents. *Am J Hum Genet* 2000;66:84–91.
- 40 Coppin L, Grutzmacher C, Crépin M, Destailleur E, Giraud S, Cardot-Bauters C, Porchet N, Pigny P. VHL mosaicism can be detected by clinical next-generation sequencing and is not restricted to patients with a mild phenotype. *Eur J Hum Genet* 2014;22:1149–52.